植 物 分 类 与 资 源 学 报 2015, 37 (2): 145~156
Plant Diversity and Resources DOI: 10.7677/ynzwyj201514070

蛋白质水平解析高山嵩草对青藏高原昼夜环境的响应*

李 雄[1,2,3], 杨云强[1,2,3], 杨时海[3,4], 马 岚[1,2,3],
孔祥翔[1,2,3], 胡向阳[1,2], 杨永平[1,2**]

(1 中国科学院昆明植物研究所东亚植物多样性与生物地理学重点实验室, 昆明 650201; 2 中国科学院昆明植物研究所中国西南野生生物种质资源库, 昆明 650201; 3 中国科学院大学, 北京 100049; 4 中国科学院青藏高原研究所, 北京 100101)

摘要: 高山嵩草 (*Kobresia pygmaea*) 是高寒草甸的重要建群种, 其生长发育同时受到年际、季节和昼夜环境变化的影响, 但目前对高山嵩草响应昼夜环境变化的研究很少。本研究通过差异蛋白质组学的方法, 结合抗氧化酶活性测定和蛋白质免疫印迹技术, 分析了高山嵩草在一天中每 4 个小时的蛋白质表达变化。结果表明: 在白天的高温、强光和紫外辐射, 以及夜里的低温等不利条件下, 高山嵩草体内的抗氧化酶、热休克蛋白和脱落酸代谢相关的蛋白质等能够被大量诱导表达, 从而对细胞和机体起到保护作用。同时, 受蛋白质调控的一些生命活动如光合作用会集中在较为适宜的时间段进行。通过体内蛋白质表达的可塑性和灵活性, 高山嵩草能够有效地应对短时间里环境的变化。

关键词: 高山嵩草; 青藏高原; 昼夜环境; 蛋白质组学; 抗氧化酶
中图分类号: Q 945.79 **文献标志码**: A **文章编号**: 2095-0845(2015)02-145-12

Protein Level Analysis of *Kobresia pygmaea* (Cyperaceae) Response to Diurnal Environment on the Tibetan Plateau

LI Xiong[1,2,3], YANG Yun-qiang[1,2,3], YANG Shi-hai[3,4], MA Lan[1,2,3],
KONG Xiang-xiang[1,2,3], HU Xiang-yang[1,2], YANG Yong-ping[1,2**]

(1 *Key Laboratory for Plant Diversity and Biogeography of East Asia*, *Kunming Institute of Botany*, *Chinese Academy of Sciences*, Kunming 650201, China; 2 *Germplasm Bank of Wild Species in Southwest China*, *Kunming Institute of Botany*, *Chinese Academy of Sciences*, Kunming 650201, China; 3 *University of Chinese Academy of Sciences*, Beijing 100049, China; 4 *Institute of Tibetan Plateau Research*, *Chinese Academy of Sciences*, Beijing 100101, China)

Abstract: *Kobresia pygmaea* is an important constructive species of alpine meadow on the Tibetan Plateau; its growth and development is influenced by both drastic environments of seasonal or inter-annual replacement and diurnal cycle. For a long time, studies about *K. pygmaea* adaption to alpine environment were mainly focused on the long-term adaptation while the diurnal responses were rarely reported. In the present study, we performed comparative proteomics approach, together with antioxidant enzyme assays and western blot, to analyze the variation of proteins expression in *K. pygmaea* every four hours from 2 a. m. to 22 p. m. in a day, which were collected from elevation of 4 800 m on the Nyainqentanglha Mountains. The results implicated that *K. pygmaea* was subjected to time-period abiotic environmental stresses, including high temperature, intense light and ultraviolet radiation in the day and low temperature in the night. To maintain normal life activities, *K. pygmaea* formed a complex set of strategies to deal with the potential damage. These strategies at least contained the plasticity and flexibility of antioxidant system, heat

* Funding: The National Natural Sciences Foundation of China (31170256) and the Major State Basic Research Development Program (2010CB951700)
** Author for correspondence; E-mail: yangyp@mail.kib.ac.cn
Received date: 2014-04-30, Accepted date: 2014-07-17
作者简介: 李雄 (1987-) 男, 博士研究生, 主要从事植物生理生态学和分子生物学研究。E-mail: lixiong@mail.kib.ac.cn

shock proteins accumulation, and abscisic acid metabolism. Meanwhile, a potential way named time-special activities regulated by proteins was also used to improve efficiency of survival, which meant some important biological processes, such as energy metabolism and photosynthesis, mostly occurred at more suitable time to avoid disadvantageous periods. These results supplied more knowledge about alpine plants adaptation to extreme day and night on the Tibetan Plateau.

Key words: *Kobresia pygmaea*; Tibetan Plateau; Diurnal environment; Proteomics; Antioxidant enzyme

The Tibetan Plateau, with a mean altitude of more than 4 000 m, is considered as roof of the world. Because of the special geographical location and complex terrain, it becomes the sensitive area and promoter region of climate change (Wang *et al.*, 2010). With global warming and the ozone weakening, researches about alpine plants response and adaption to environmental factors become more focused hotspots. In addition, the Tibetan Plateau forms typical alpine environment with large temperature change between day and night, intense solar radiation, and some other environmental factors, which are considered to be harsh environment for plants (Zhang *et al.*, 2010). Therefore, alpine plants growing here must have gained special structural characteristics and physiological, ecological adaptation tactics.

Environmental change includes long-term seasonal or inter-annual variation and short-time diurnal turnover. Alpine environments usually show more intense and complex changes. Plants, especially at high altitudes, need to accommodate both long-term and short-time environmental changes to complete normal growth and development. Although the topic of plants adaption to environment has attracted great attentions all the time, studies were almost reflected in the long-term adaptation in the wild (Annicchiarico *et al.*, 1995; Yang *et al.*, 2011; Yang *et al.*, 2012). The situations of plants response to diurnal variation were mainly focused on circadian clock regulation to the diurnal rhythm in model plant, like *Arabidopsis thaliana*, under laboratory conditions (Wang and Tobin, 1998; Somers, 1999; Doyle *et al.*, 2002; Farre *et al.*, 2005; Pruneda-Paz and Kay, 2010; Lai *et al.*, 2012). However, relevant studies were lack of verification in natural field, not to mention the Tibetan Plateau.

Alpine meadow, one widely distributed vegetation type on Tibetan Plateau, is the product of alpine climate. In alpine meadow, *Kobresia pygmaea*, a member of sedge family, often acts as the constructive species, which is mainly propagated by vegetations because of the very low seed setting rate and germination rate (Li *et al.*, 2013). *K. pygmaea* plays a crucial role in maintaining the stability of the regional ecology environment, since it has a strong viability with many excellent features, such as resistance to low temperature, drought, trampling and soil erosion (Miehe *et al.*, 2008). Therefore, the researches about how *K. pygmaea* adapts to alpine environment and responds to global climate change are very significant. At present, relevant studies were mainly focused on the long-term adaptation primarily from the morphological structure (Yang *et al.*, 2011) and physiological aspects (Yang *et al.*, 2012), but the response to diurnal environmental change has rarely been reported. In this study, we adopted comparative proteomics approach, together with antioxidant enzyme activity assays and western blot to analyze the variations of proteins expression of *K. pygmaea* in a day. The results can deepen our understanding alpine plants adaptation to extreme day and night on the Tibetan Plateau.

1 Material and methods

1.1 Samples collection

This study was conducted in July, 2012, on the south-facing slope of the Nyainqentanglha Mountains on altitude of 4 800 m (30°31′53″ N, 91°03′18″ E) near Damxung City in central Tibetan Plateau. To analyze internal response of *K. pygmaea* to external

environment in a day, we collected samples at Zeitgeber time (ZT) 2 (2 a. m.), ZT6 (6 a. m.), ZT10 (10 a. m.), ZT14 (14 p. m.), ZT 18 (18 p. m.) and ZT22 (22 p. m.) respectively. At each time point, 100 to 200 g healthy leaves of *K. pygmaea* from a 25-m^2 region were randomly selected and immediately frozen by liquid nitrogen for later protein extraction and enzyme assays. The samples were collected and the experiments were conducted in triplicate.

1.2 Detection of environmental factors

The environmental factors including temperature, light intensity, ultraviolet (UV) radiation and atmospheric humidity at different time were observed and measured.

1.3 Antioxidant enzyme assays

Approximately 1 g of leaves from each sample collected at different time were homogenized in extraction buffer (50 mmol · L^{-1} sodium phosphate pH 7.0, 1 mmol · L^{-1} EDTA, 1 mmol · L^{-1} DTT, 1 mmol · L^{-1} GSH, 1 mmol · L^{-1} ASA, 5 mmol · L^{-1} $MgCl_2 \cdot 6H_2O$, 1% PVP-40 and 20% glycerin) as the ratio of 100 mg tissue/mL buffer. The homogenates were centrifuged at 12 000 × *g* for 15 min at 4 ℃, and the total soluble protein contents in the supernatants were measured according to the Bradford method (Barbosa *et al.*, 2009). The activities of catalase (CAT, EC1.11.1.6), ascorbate peroxidase (APX, EC1.11.1.11), superoxide dismutase (SOD, EC1.15.1.1), and glutathione reductase (GR, EC1.6.4.2) were determined as previously applied method (Beaucham and Fridovic, 1971; Nakano and AsAda, 1981; Varga *et al.*, 2012).

1.4 Protein extraction and two-dimensional gel electrophoresis

Protein extraction and 2D separation was performed according to the previous methods (Damerval *et al.*, 1986), with some modifications. Approximately 10−20 g leaves from each sample collected at different time were grounded in liquid nitrogen and total soluble proteins were extracted on ice in acetone containing 10% trichloroacetic acid (TCA) and 0.07% DTT. The homogenates were placed at −20 ℃ for 4 h and then were centrifuged (8 000 × *g*, 30 min, 4 ℃). The resulted pellets were washed with acetone containing 0.07% DTT at −20 ℃ for 30 min and then centrifuged (8 000 × *g*, 20 min, 4 ℃), which was repeated for 3 times. The final pellets were vacuum-dried and then dissolved in lysate (7 M urea, 2 M thiourea, 4% CHAPS and 60 mmol · L^{-1} DTT) for 2 h at room temperature with intermittently shocking, and then the samples were centrifuged (12 000 × *g*, 20 min, 20 ℃). The supernatants were collected for 2-DE experiments with 900 μg of total proteins by a method used previously (Bai *et al.*, 2011), which were executed in triplicate.

1.5 Spots digestion and protein identification for Mass Spectrometry analyses

Protein spots that showed significant changes in expression with change in elevation were excised manually from colloidal CBB-stained 2-DE gels. Protein digestion with trypsin was first performed; then mass spectrometry analyses were conducted using a MALDI-TOF/TOF mass spectrometer 4 800-plus Proteomics Analyzer (Applied Biosystems, Farmington, MA, USA) according to methods previously described (Bai *et al.*, 2011).

1.6 Database search

The primary and secondary MS data were transferred into Excel files and used as inputs to search against an NCBI non-redundant database; the search was restricted to viridiplantae (green plants) using the MASCOT search engine (www.matrixscience.com). The search parameters were established as follows: no restriction of protein molecular weight; one missed trypsin cleavage allowed; cysteine treated by iodoacetamide; and oxidation of methionine. The peptide tolerance was 100 ppm and the MS/MS tolerance was 0.25 kD. Protein identifications were validated manually, with at least four peptides matching. The keratin contamination was removed and the MOWSE score threshold was greater than 40 ($P < 0.05$). Only significant hits were accepted for the identification of the protein sample based on MASCOT probability analysis.

1.7 Western blot analysis

SDS-PAGE injected with equal amount of total protein was performed as a reference to previous method (Laemmli *et al.*, 1970) using 12% polyacrylamide slab gels. Protein samples were electroblotted onto polyinylidene difluoride (PVDF) membranes by using a Trans-Blot cell (Bio-Rad) for western blot analysis. After transfer, the membranes were blocked for 1 h at room temperature. Membranes were probed with the appropriate primary antibodies and HRP-conjugated goat anti-rabbit secondary antibody (Promega, Madison, WI 53711, US), and signals were detected using an ECL kit (GE Company, Evansville, Indiana 47715, US). The primary antibodies were diluted as follows: 9-cis-epoxycarotenoid dioxygenase (NCED) antibody, 1∶1000; dehydrin antibody, 1∶1000.

2 Results and discussion

2.1 Abiotic environment factors incite relational protein expression: proteome analysis

To investigate the response of *K. pygmaea* to diurnal environment change in protein level, we performed 2-DE to identify the whole protein accumulation profile in *K. pygmaea* from ZT2 to ZT22. We performed three biological replicates and gels were visualized by CBB staining (Fig. S1, S2, S3). After staining, proteins were analyzed by PDQuest software (Bio-Rad). Under stringent condition, all differentially displayed proteins were unambiguously identified by MALDI-TOF-MS/MS analysis and searched against the NCBI nonredundant database. In total, comparative proteomics patterns of six different time samples indicated that the expressions of 120 detectable proteins varied by at least 1.5-fold ($P < 0.05$) and 60 of these protein spots were positively identified using MALDI-TOF MS (Table S1). According to the NCBI annotations, the identified proteins could be classified into six functional groups: Oxidation reduction processes, stress resistance, energy metabolism, photosynthesis, biosynthesis and metabolism and others (Fig. 1A). Among these protein spots, we found that the stress resistance, photosynthesis and oxidation reduction processes consisted of most of the identified proteins, occupying 22%, 18% and 18% of all respectively (Fig. 1A). A hierarchical cluster analysis was conducted to categorize the proteins that showed differential expression profiles at each time (Fig. 1B).

The study demonstrated that stress resistance proteins response to cold, heat or light and some proteins involved in oxidation reduction processes showed significant changes in expression with diurnal cycle. In particular, a group of important proteins belonging to the antioxidant system, such as ascorbate peroxidase (spot 135) and manganese superoxide dismutase (spot 147) exhibited significant oscillations (Fig. 2, Fig. 1B, C), which exhibited greater expression at ZT6 or ZT14, corresponding to the extreme values of environmental factors.

Heat shock proteins (HSPs), well known in all eukaryotic organisms, play essential roles in various cellular processes when plants are exposed to stressful conditions, such as high or low temperatures, oxygen deprivation, etc. (Siaussat *et al.*, 2013) Under high temperature, HSPs produced in plants can protect organism proteins from damaging or repair damaged proteins, indicating that induced formation of heat shock protein make plant to acquire heat resistance (Kato *et al.*, 1993). In this study, we detected four heat shock-related proteins: putative heat-shock protein (spot 3), heat shock protein, putative (spot 8), stromal 70 kDa heat shock-related protein (spot 9) and heat shock protein hsp20 (spot 173). Three of these proteins showed expression peak at ZT14, and the other one peaked at ZT10 (Fig. 2, Fig. 1B). The results fully demonstrated that high temperature in the afternoon (Table S1) was an unfavorable factor for *K. pygmaea*, so large amount of heat shock proteins were induced to play a potential protective effect.

Light is the necessary condition of photosynthesis for plants. In the day light, especially at ZT10, proteins related to light harvesting or light stimulation dis-

played a higher expression (Fig. 1B). It may be crucial for the growth of *K. pygmaea* due to volatile environment conditions. For example, in the afternoon, intense light accompanied by high temperature may disturb photosynthesis. Taken together, proteins response to temperature or light, the major abiotic environment factors in diurnal cycle, were incited at special time when they became dominant factors (Table S1).

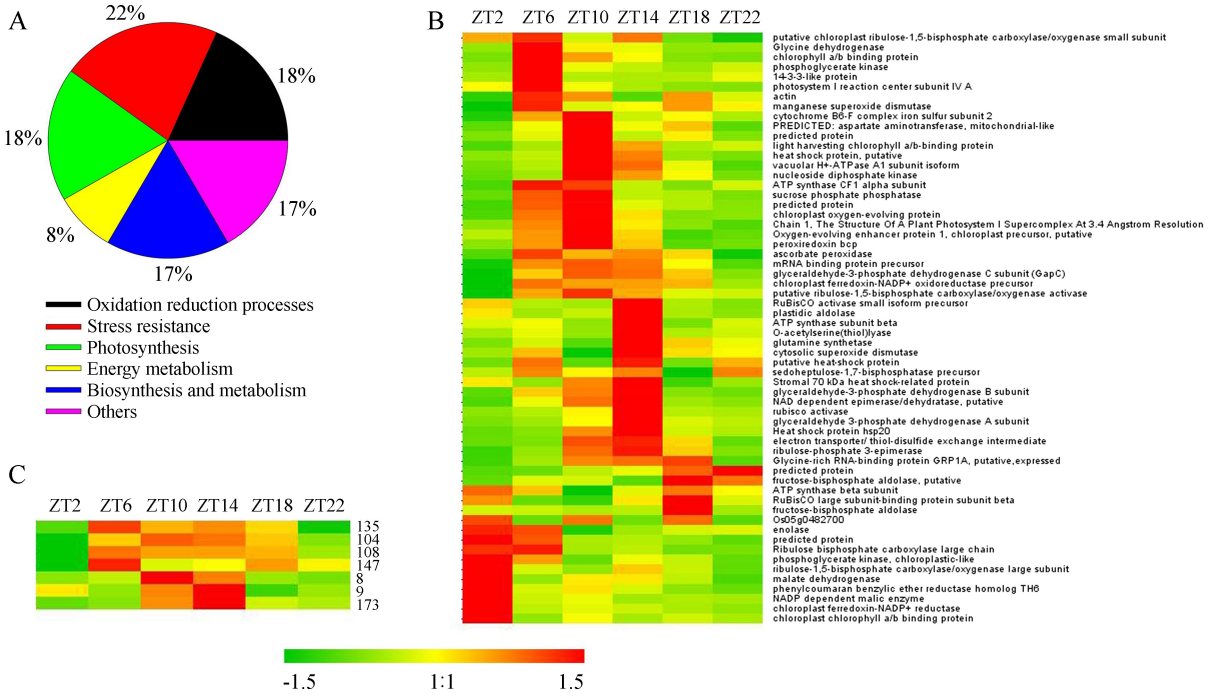

Fig. 1　Functional classification and hierarchical clustering of the identified proteins

A. Functional classification of the identified proteins; B. Hierarchical clustering of the identified protein expression profiles at different times; C. Hierarchical clustering of some important proteins mentioned in Fig. 2. Different colors correspond to the proteins' log-transformed fold-change ratios depicted in the bar at the bottom of the figure

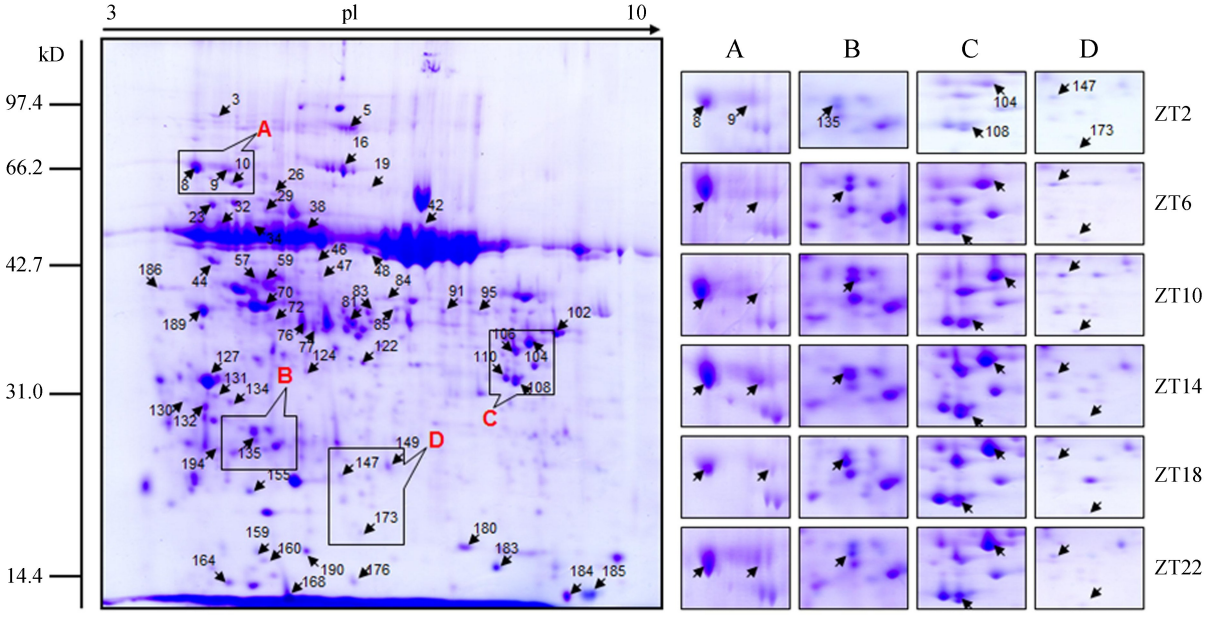

Fig. 2　Representative 2D gel of total protein from plants at ZT14 (left) and enlarged windows (A-D) of gel (right) shown on left of plants from different time. The numbers assigned to the protein spots correspond to those listed in Table S1

2.2 Expressions of functional group proteins were aggravated at specific times of the day

Based on the functional classification of comparative proteins, we investigated the expression patterns of group proteins with analogous function. The number of proteins with expressing peak or trough at each time of six functional groups was counted separately (Fig. 3). Indeed, nearly all groups showed the homologous results that proteins expression were elevated at specific times of the day and simultaneously inhibited at some other times. More specifically, proteins involved in stress resistance mainly peaked from ZT2 to ZT14, with significant difference at ZT14 (Fig. 3A). This illustrated that ZT2 to ZT14 was the adverse period of *K. pygmaea*, especially at ZT14 accompanied by high temperature and intense radiation. Nevertheless, we also found that some resistance proteins reached trough level from ZT18 to ZT2, exhibiting significant difference at ZT2 (Fig. 3A). These results manifested that some proteins were restrained in the evening or night, while those involved in cold resistance were stated at the same time. Oxidation reduction processes are a set of important courses along with the resilience reaction of plants. Likewise, the expression of proteins related to redox processes reached the maximum from ZT2 to ZT14 and fell to the bottom from ZT18 to ZT2. The difference was that the largest number appeared at ZT6 and ZT18 respectively (Fig. 3B). Interestingly, we observed analogous appearance on proteins involved in energy metabolism (Fig. 3C). Collectively, above results suggested that these three processes, resilience reaction, redox process and energy metabolism, were a series of interrelated complex processes in *K. pygmaea*. By doing this, the plants can integrate information between physiological metabolism and environmental change to withstand abiotic challenges.

Apart from above results, energy metabolism, together with biosynthesis and metabolism, are closely contacted with plants photosynthesis. Proteins involved in photosynthesis were more active in the daytime (Fig. 1B), when the light and temperature was relatively suitable. Precisely, overwhelming majority proteins were animated at ZT10 compared with small amount at ZT14 (Fig. 3D). This reveled not only that maybe conditions at noon were the most suitable for *K. pygmaea*, but also that photosynthesis was under suppression in the afternoon. Previous studies have certificated that strong light (Poulson *et al.*, 2006), high temperature (Zhou *et al.*, 2010; Yu *et al.*, 2013) and UV radiation (Lud *et al.*, 2002) obstruct photosynthesis through complex mechanism; nevertheless, plants also have evolved a set of defense mechanisms (Liu *et al.*, 2012). Low temperature is regarded as an important interference factor for many physiological processes, and photosynthesis is one of the most obvious processes affected by low temperature (Oquist *et al.*, 1993; Liu *et al.*, 2012). This is why many proteins with various functions exhibited expression trough at midnight (Fig. 3). As photosynthesis is essential for plants, in natural environment, the strategic that efficient photosynthesis concentrated in a short time is highly beneficial to *K. pygmaea* in the long term. Extreme expression of proteins involved in biosynthesis and metabolism (Fig. 3E) and some other functions (Fig. 3F) were observed in each time without significant difference, indicating participation throughout the whole day. In summary, expressions of functional group proteins were exacerbated at appropriate times of the day in this research. The temporal allocation may be favorable for *K. pygmaea* to maintain normal physiological activities in the complicated and changeable environment.

2.3 Antioxidant enzyme activities exhibited changes in volatility

Plants will produce reactive oxygen species (ROS) when undergone aerobic metabolism, e. g., photosynthesis and respiration (Apel and Hirt, 2004). If the generation of ROS is not removed timely, plants may experience oxidative stress due to breaking the homeostasis in cellular redox state that may eventually lead to cell death (Apel and Hirt, 2004). Thus, plants have evolved scavenging machineries with an-

tioxidant enzymes and antioxidants to keep ROS at physiologically permissive levels (Mittler, 2002).

Here we determined the activities of four antioxidant enzymes (CAT, APX, SOD, and GR) to investigate the response of *K. pygmaea* to the complex external environment. We observed that all four enzyme activities exhibited similarly fluctuation in the day (Fig. 4), which peaked from ZT2 to ZT6 or from ZT14 to ZT18 and dipped at ZT10 or ZT22. Interestingly, the results were identical to proteomics results of antioxidant system observed above. Our results are consistent with previous reports that some antioxidant enzyme activities, such as APX, GR, and SOD increased under low temperature or heat stress (Lee and Lee, 2000; Lou *et al.*, 2011; Liu *et al.*, 2012; Yu *et al.*, 2013). Thus, the results suggested unfriendly abiotic environment e. g., cold in the mid-night, heat and UV radiation in the afternoon, could induce the production of ROS in *K. pygmaea*. To prevent the potential threat, plants can improve corresponding antioxidant enzyme activities to keep ROS homeostasis.

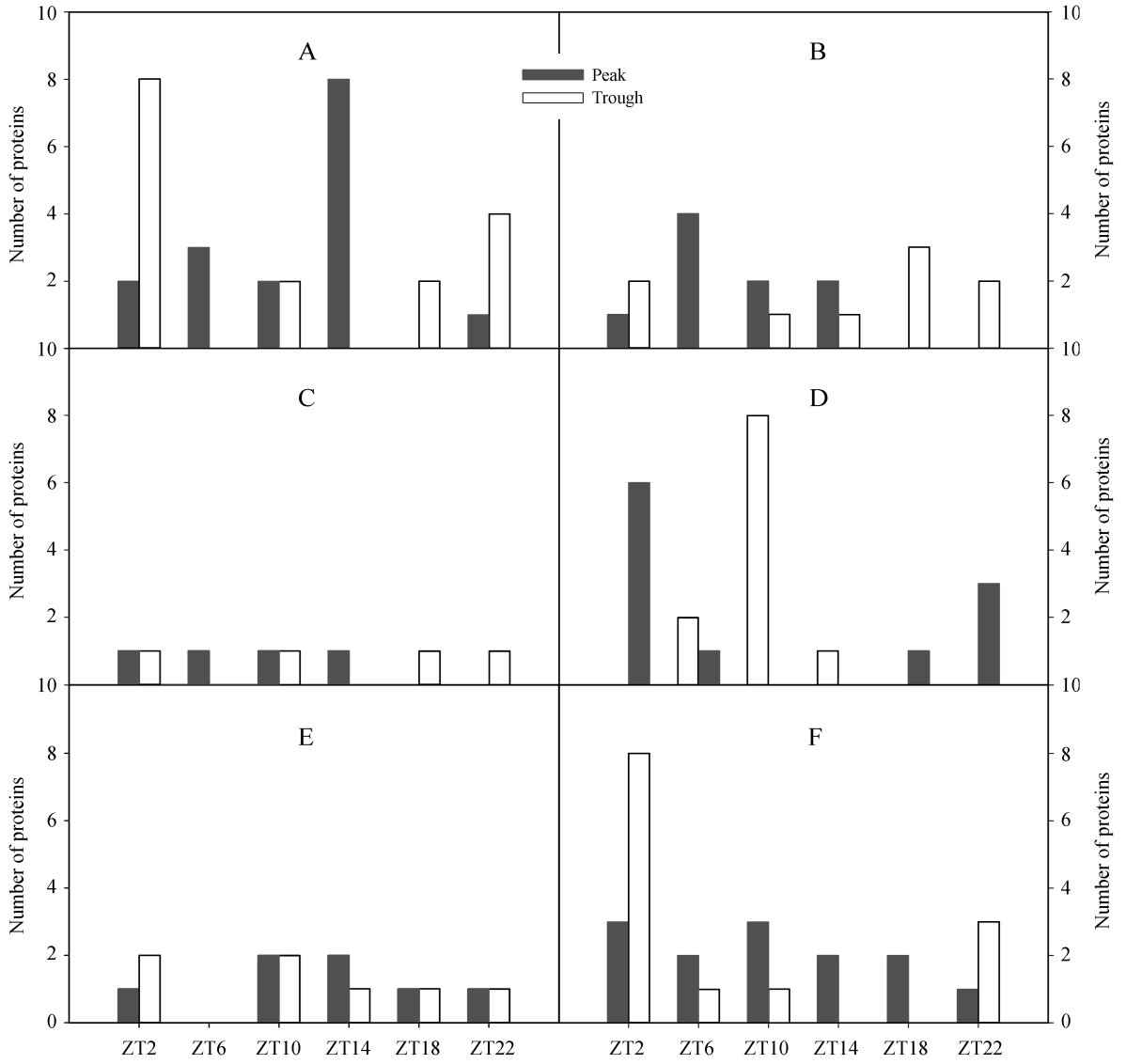

Fig. 3　The number of proteins expressing at peak or trough according to the functional classification in Fig. 1A at different times

A. Stress resistance; B. Oxidation reduction processes; C. Energy metabolism; D. Photosynthesis; E. Biosynthesis and metabolism; F. Others

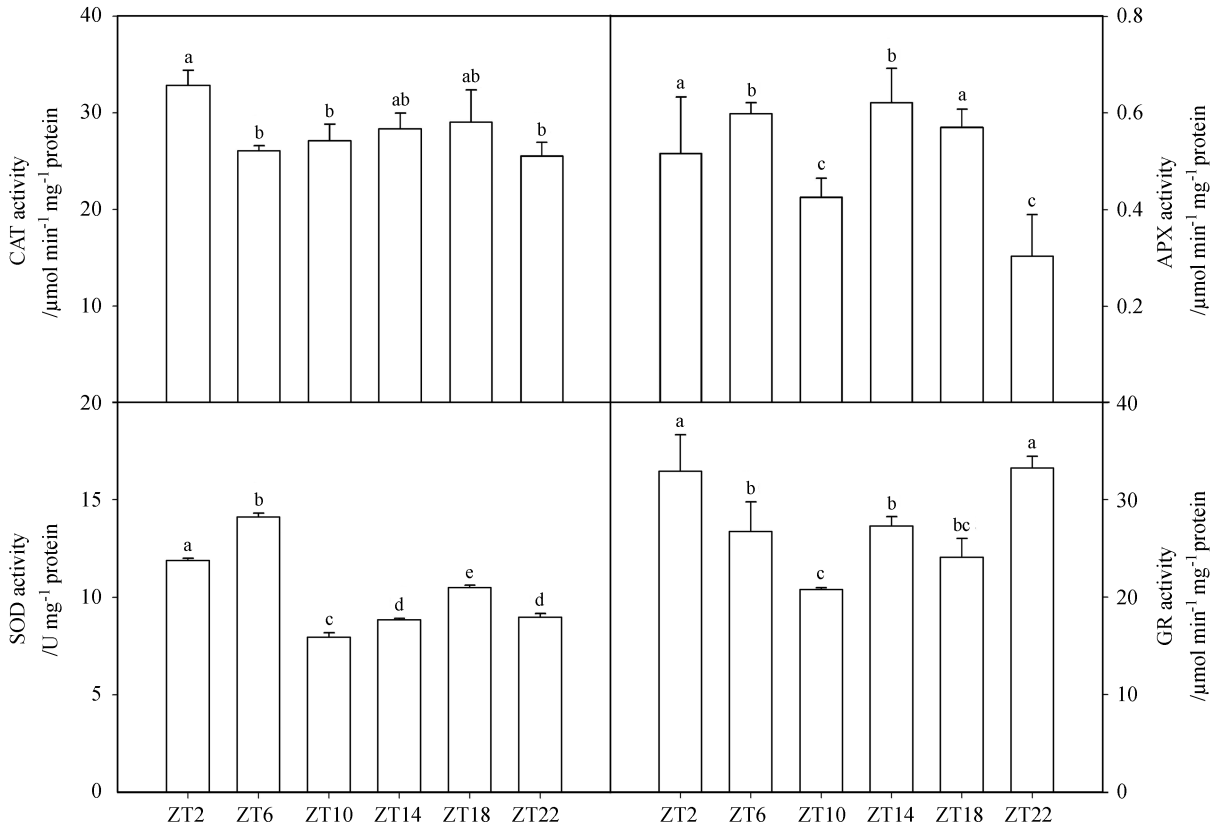

Fig. 4 The change of antioxidant enzyme activities in *Kobresia pygmaea* at different times. Different symbols indicate significant differences between treatments ($P<0.05$) according to Tukey's test

Previous study reported that circadian clock regulated ROS production and scavenging in *Arabidopsis thaliana* (Lai *et al.*, 2012), and a core feedback loop of circadian clock has been found in plants (Wang and Tobin, 1998; Alabadi *et al.*, 2001; Pruneda-Paz and Kay, 2010). We hypothesized that antioxidant enzyme activities of *K. pygmaea* also would be regulated by circadian clock, despite lack of appropriate mechanism. Nevertheless, plants in natural field, especially on Qinghai-Tibet Plateau, may suffer sudden stress from unstable environment factors, resulting in irregular fluctuation of ROS production. In the present study, we confirmed the truth of that *K. pygmaea* coordinated ROS metabolic processes with the external environment to avoid damaging the plants.

2.4 Abscisic acid and dehydrin might participate in a variety of physiological regulation

To detect some substances played important roles in plant life activities from the perspective of protein, we performed immunoblot analysis with specific antibodies against abscisic acid (ABA) synthase and dehydrin. ABA is an important plant hormone modulating seed dormancy, germination, stoma closure and responses to abiotic stresses, including low temperatures, drought and high temperature (Fujita *et al.*, 2006). Its synthesis is mediated by ABA synthases, like 9-cis-epoxycarotenoid dioxygenase (NCED) in plants. Dehydrins, belonging to a multi-family of proteins, are present in plants and induced by cold, salt, ABA and drought stress (Kosova *et al.*, 2011). In the results of western blot, we found NCED was highly accumulated at ZT2, ZT6, ZT14, ZT18 and ZT22, but with little expression at ZT10 (Fig. 5), implying similar regularity about production of ABA. Combined with environmental change, the result signified ABA was involved in *K. pygmaea* resistance to cold, heat and UV radia-

tion. In spite of lower expression at ZT14 compared with ZT2, ABA was also induced in the afternoon by heat to regulate stomas closure, may be this partly explained many proteins related to photosynthesis were down-regulated. As for dehydrins, we observed the same expression variation (Fig. 5). This suggested dehydrins were partly induced by ABA and played important roles in response to extreme conditions. Overall, we found the ABA synthase and dehydrin were altered with external conditions, which insinuated they were involved in a variety of physiological regulation. Furthermore, the fact that both ABA synthase and dehydrin displayed minimal expression at ZT10 indicated this time might be the more appropriate condition for *K. pygmaea* as regarded above once again.

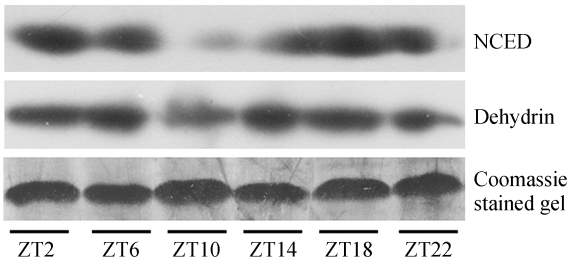

Fig. 5　The effect of environment change on the protein accumulation of abscisic acid synthase and dehydrin for the six samples from different time. The coomassie stained gel is included as a protein loading control

3　Conclusions

In the present study, we investigated and discussed the mode of *K. pygmaea* response to the natural environment during day and night in the protein level. Several categories of proteins were particularly noticed to show apparent volatility changes with the ups and downs of environmental factors, which exhibited strong plasticity and flexibility. By analyzing the intrinsic link, we found that *K. pygmaea* might suffer from time period abiotic environmental stresses, including high temperature, intense light and UV radiation in the daytime and low temperature in the night during a diurnal cycle. To maintain normal life activities, *K. pygmaea* would not stand still and formed a complex set of strategies to fight against the potential damage. These strategies at least contained antioxidant system, HSPs accumulation and ABA metabolism. In addition, a lot of important activities, such as energy metabolism and photosynthesis, mostly occurred at more suitable time to avoid disadvantageous periods, which were designated as time-special activities. Briefly, the plasticity and flexibility of some functional proteins was the material basis of *K. pygmaea* adaptation to the large diurnal environmental changes. As there was little attention that had been paid to *K. pygmaea* at the molecular level before (Li *et al.*, 2013), our results could improve understanding of the interaction between alpine plants and natural environment.

References:

Alabadi D, Oyama T, Yanovsky MJ *et al.*, 2001. Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis diurnal clock [J]. *Science*, **293**: 880—883

Annicchiarico P, Bozzo F, Parente G *et al.*, 1995. Analysis of adaptation of grass/legume mixtures to Italian alpine and subalpine zones through an additive main effects and multiplicative interaction model [J]. *Grass and Forage Science*, **50**: 405—413

Apel K, Hirt H, 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction [J]. *Annual Review of Plant Biology*, **55**: 373—399

Bai XG, Yang LM, Yang YQ *et al.*, 2011. Deciphering the protective role of nitric oxide against salt stress at the physiological and proteomic levels in maize [J]. *Journal of Proteome Research*, **10**: 4349—4364

Barbosa H, Slater NKH, Marcos JC, 2009. Protein quantification in the presence of poly (ethylene glycol) and dextran using the Bradford method [J]. *Analytical Biochemistry*, **395**: 108—110

Beaucham C, Fridovic I, 1971. Superoxide dismutase-improved assays and an assay applicable to acrylamide gels [J]. *Analytical Biochemistry*, **44**: 276—287

Damerval C, Devienne D, Zivy M *et al.*, 1986. Technical improvements in two-dimensional electrophoresis increase the level of genetic-variation detected in wheat-seedling proteins [J]. *Electrophoresis*, **7**: 52—54

Doyle MR, Davis SJ, Bastow RM *et al.*, 2002. The ELF4 gene controls diurnal rhythms and flowering time in *Arabidopsis thaliana* [J]. *Nature*, **419**: 74—77

Farre EM, Harmer SL, Harmon FG *et al.*, 2005. Overlapping and distinct roles of PRR7 and PRR9 in the Arabidopsis diurnal clock [J]. *Current Biology*, **15**: 47—54

Fujita M, Fujita Y, Noutoshi Y *et al.*, 2006. Crosstalk between abiot-

ic and biotic stress responses: a current view from the points of convergence in the stress signaling networks [J]. *Current Opinion in Plant Biology*, **9**: 436—442

Kato S, Yamagishi K, Tatsuzawa F *et al.*, 1993. Identification of cytoplasmic and nuclear low-molecular-weight heat-shock proteins in tomato fruit [J]. *Plant and Cell Physiology*, **34**: 367—370

Kosova K, Vitamvas P, Prasil IT, 2011. Expression of dehydrins in wheat and barley under different temperatures [J]. *Plant Science*, **180**: 46—52

Laemmli UK, Beguin F, Gujerkel G, 1970. A factor preventing major head protein of bacteriophage T4 from random aggregation [J]. *Journal of Molecular Biology*, **47**: 69—74

Lai AG, Doherty CJ, Mueller-Roeber B *et al.*, 2012. DIURNAL CLOCK-ASSOCIATED 1 regulates ROS homeostasis and oxidative stress responses [J]. *Proceedings of the National Academy of Sciences of the United States of America*, **109**: 17129—17134

Lee DH, Lee CB, 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays [J]. *Plant Science*, **159**: 75—85

Li X, Hu XY, Yang YP, 2013. Research of important forage *Kobresia pygmaea* in Qinghai-Tibet Plateau [J]. *Pruataculture & Animal Husbandry*, **1**: 30—39

Liu YF, Qi MF, Li TL, 2012. Photosynthesis, photoinhibition, and antioxidant system in tomato leaves stressed by low night temperature and their subsequent recovery [J]. *Plant Science*, **196**: 8—17

Lou B, Xu DD, Xu HX *et al.*, 2011. Effect of high water temperature on growth, survival and antioxidant enzyme activities in the Japanese flounder *Paralichthys olivaceus* [J]. *African Journal of Agricultural Research*, **6**: 2875—2882

Lud D, Moerdijk TCW, Van de Poll WH *et al.*, 2002. DNA damage and photosynthesis in Antarctic and Arctic *Sanionia uncinata* (Hedw.) Loeske under ambient and enhanced levels of UV-B radiation [J]. *Plant Cell and Environment*, **25**: 1579—1589

Miehe G, Mlehe S, Kaiser K *et al.*, 2008. Status and dynamics of *Kobresia pygmaea* ecosystem on the Tibetan plateau [J]. *Ambio*, **37**: 272—279

Mittler R, 2002. Oxidative stress, antioxidants and stress tolerance [J]. *Trends in Plant Science*, **7**: 405—410

Nakano Y, AsAda K, 1981. Hydrogen-peroxide is scavenged by ascorbate-specific peroxidase in spinach-chloroplasts [J]. *Plant and Cell Physiology*, **22**: 867—880

Oquist G, Hurry VM, Huner NPA, 1993. Low-temperature effects on Photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye [J]. *Plant Physiology*, **101**: 245—250

Poulson ME, Boeger MRT, Donahue RA, 2006. Response of photosynthesis to high light and drought for *Arabidopsis thaliana* grown under a UV-B enhanced light regime [J]. *Photosynthesis Research*, **90**: 79—90

Pruneda-Paz JL, Kay SA, 2010. An expanding universe of diurnal networks in higher plants [J]. *Trends in Plant Science*, **15**: 259—265

Siaussat D, Laparie M, Maria A *et al.*, 2013. Heat shock protein responses to salinity, food deprivation, and temperature in the invasive ground beetle *Merizodus soledadinus* at the Kerguelen Islands [J]. *Polar Biology*, **36**: 201—209

Somers DE, 1999. The physiology and molecular bases of the plant diurnal clock [J]. *Plant Physiology*, **121**: 9—19

Varga B, Janda T, Laszlo E *et al.*, 2012. Influence of abiotic stresses on the antioxidant enzyme activity of cereals [J]. *Acta Physiologiae Plantarum*, **34**: 849—858

Wang Y, Meng LL, Yang YP *et al.*, 2010. Change in floral orientation in *Anisodus luridus* (Solanaceae) protects pollen grains and facilitates development of fertilized ovules [J]. *American Journal of Botany*, **97**: 1618—1624

Wang ZY, Tobin EM, 1998. Constitutive expression of the DIURNAL CLOCK ASSOCIATED 1 (CCA1) gene disrupts diurnal rhythms and suppresses its own expression [J]. *Cell*, **93**: 1207—1217

Yang Y, Wang GX, Klanderud K *et al.*, 2011. Responses in leaf functional traits and resource allocation of a dominant alpine sedge (*Kobresia pygmaea*) to climate warming in the Qinghai-Tibetan Plateau permafrost region [J]. *Plant and Soil*, **349**: 377—387

Yang Y, Wang GX, Yang LD *et al.*, 2012. Physiological responses of *Kobresia pygmaea* to warming in Qinghai-Tibetan Plateau permafrost region [J]. *Acta Oecologica-International Journal of Ecology*, **39**: 109—116

Yu C, Huang SJ, Hu XM *et al.*, 2013. Changes in photosynthesis, chlorophyll fluorescence, and antioxidant enzymes of mulberry (*Morus* ssp.) in response to salinity and high-temperature stress [J]. *Biologia*, **68**: 404—413

Zhang LY, Turkington R, Tang Y, 2010. Flowering and fruiting phenology of 24 plant species on the north slope of Mt. Qomolangma (Mt. Everest) [J]. *Journal of Mountain Science*, **7**: 45—54

Zhou HH, Chen YN, Li WH *et al.*, 2010. Photosynthesis of *Populus euphratica* in relation to groundwater depths and high temperature in arid environment, northwest China [J]. *Photosynthetica*, **48**: 257—268

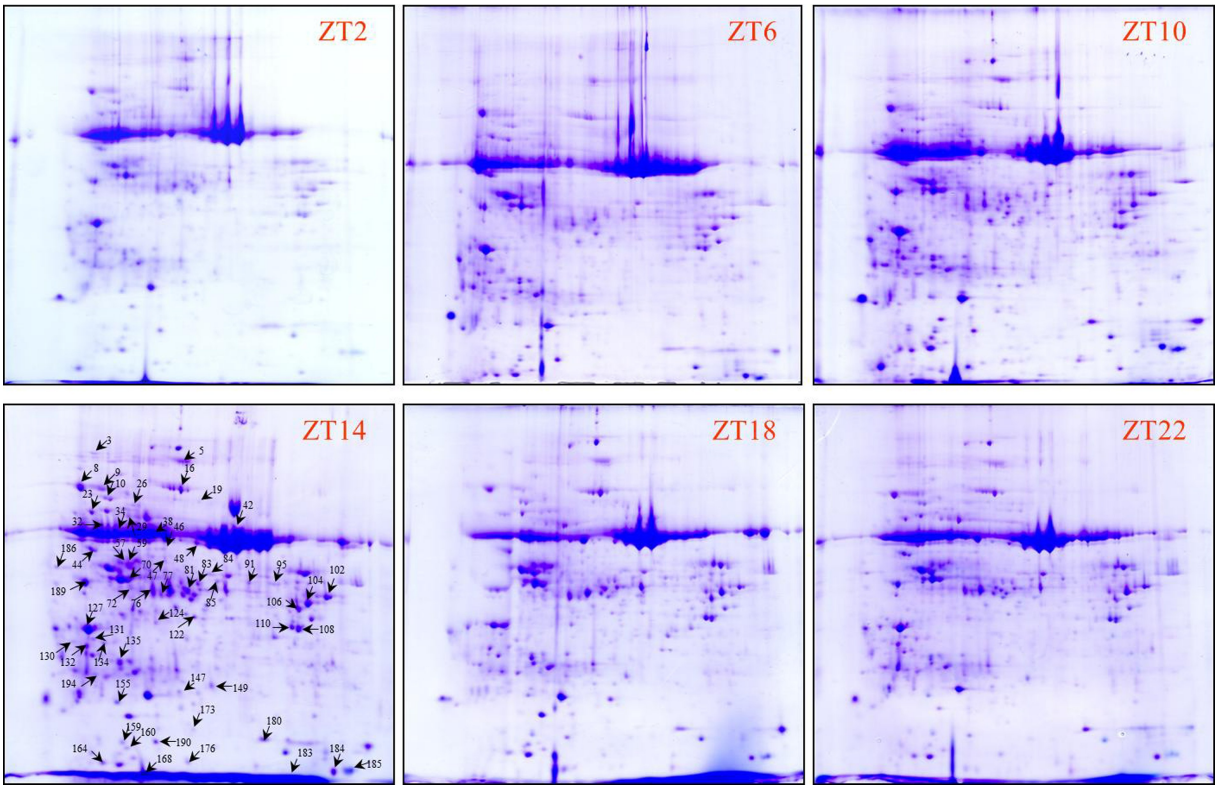

Fig. S1　The first set 2-DE of six samples from different time

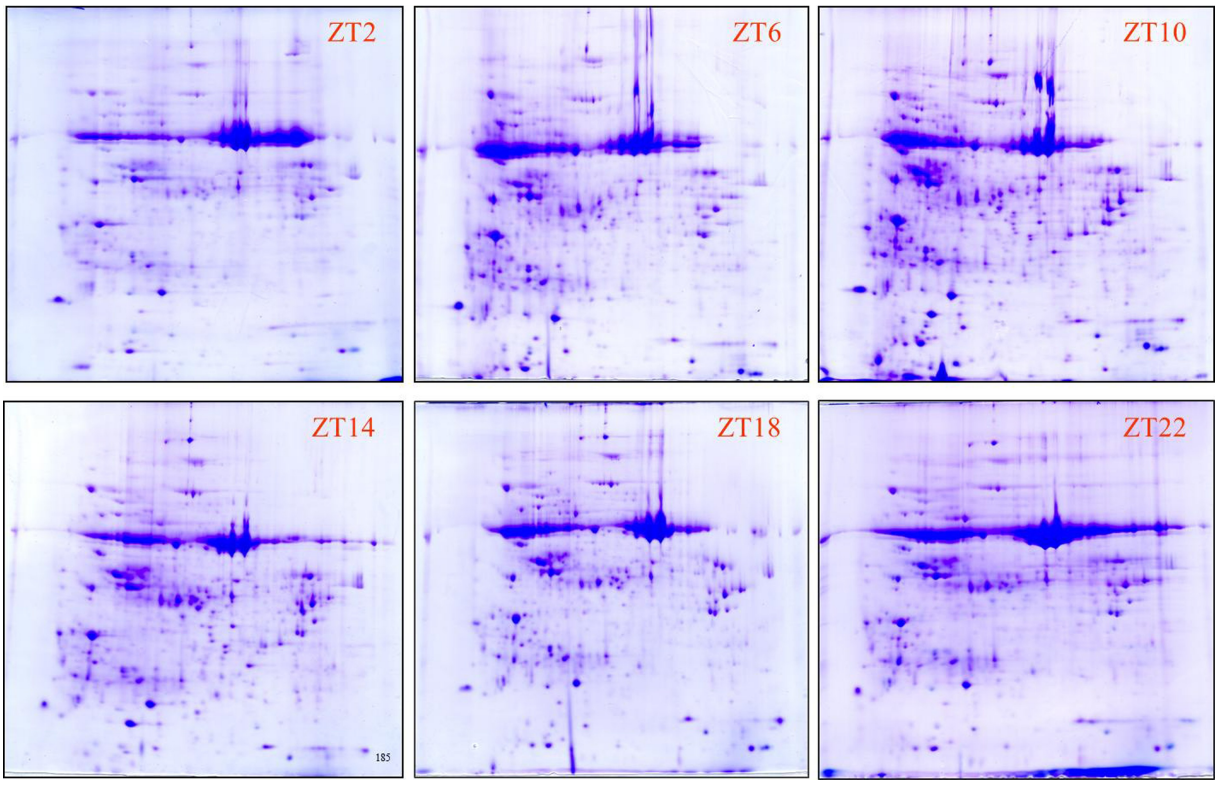

Fig. S2　The second set 2-DE of six samples from different time

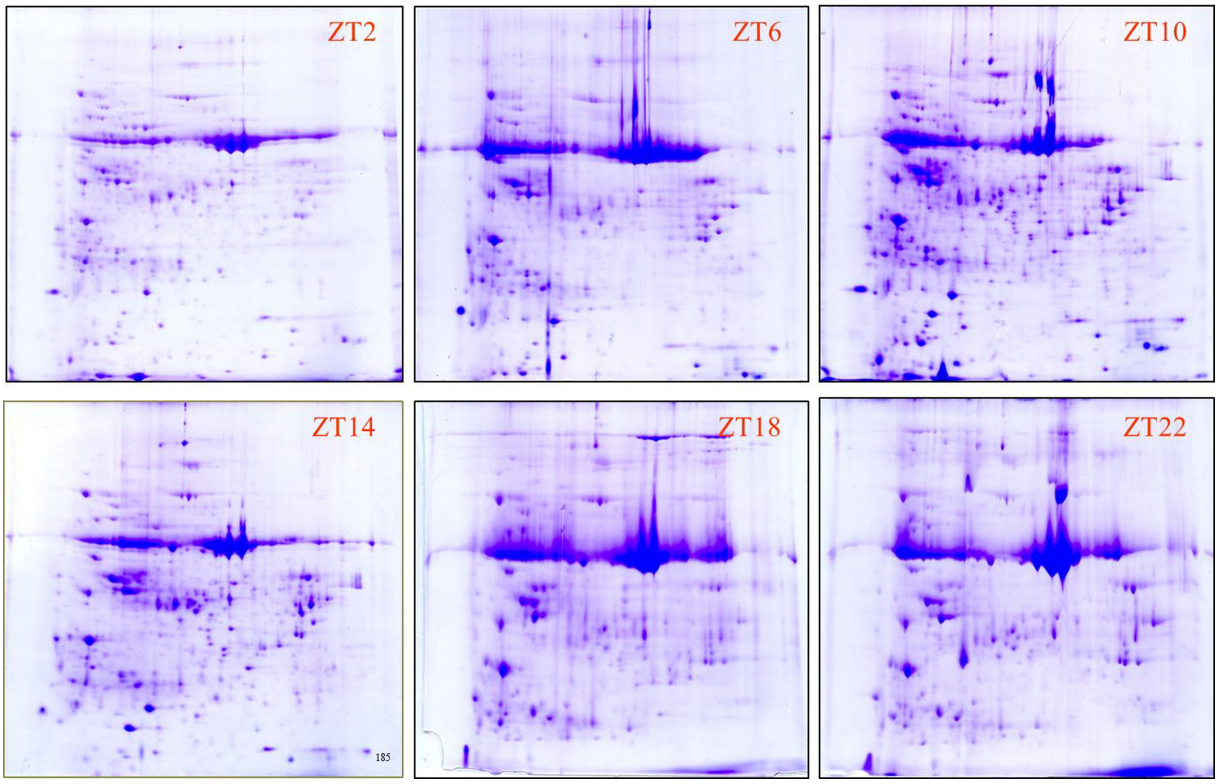

Fig. S3 The third set 2-DE of six samples from different time

Table S1 The dynamic changes of environmental factors at different time when the plant samples were collected

Symbol	Time	Temperature/℃	Light intensity	UV radiation	Atmospheric humidity
ZT2	2 a. m.	5. 00	/	/	***
ZT6	6 a. m.	4. 12	*	/	***
ZT10	10 a. m.	12. 35	**	**	**
ZT14	14 p. m.	28. 50	***	***	*
ZT18	18 p. m.	12. 50	**	**	**
ZT22	22 p. m.	6. 50	/	/	**

“/” means no existence; “ * ” means weak; “ ** ” means moderate; “ *** ” means intense